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Glutamine Enhances Selectivity of Chemotherapy Through Changes in Glutathione Metabolism

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Objective

Chemotherapy doses are limited by toxicity to normal tissues. Intravenous glutamine protects liver cells from oxidant injury by increasing intracellular glutathione (GSH) content. The authors hypothesized that supplemental oral glutamine (GLN) would increase the therapeutic index of methotrexate (MTX) by improving host tolerance through changes in glutathione metabolism. The authors examined the effects of oral glutamine on tumor and host glutathione metabolism and response to methotrexate.

Methods

Thirty-six 300-g Fischer 344 rats were implanted with fibrosarcomas. On day 21 after implantation, rats were randomized to receive isonitrogenous isocaloric diets containing 1 g/kg/day glutamine or glycine (GLY) by gavage. On day 23 after 2 days of prefeeding, rats were randomized to one of the following four groups receiving an intraperitoneal injection of methotrexate (20 mg/kg) or saline (CON): GLN + MTX, GLY + MTX, GLN-CON, or GLY-CON. On day 24, rats were killed and studied for arterial glutamine concentration, tumor volume, tumor, kidney and gut glutaminase activity, and glutathione content (tumor, gut, heart, liver, muscle, kidney, and lung).

Results

Provision of the glutamine-enriched diets to rats receiving MTX decreased tumor glutathione (2.38 ± 0.17 in GLN + MTX *vs.* 2.92 ± 0.20 in GLY + MTX, $p < 0.05$), whereas increasing or maintaining host glutathione stores (in gut, 2.60 ± 0.28 in GLN + MTX *vs.* 1.93 ± 0.18; in GLY + MTX, $p < 0.05$). Depressed glutathione levels in tumor cells increases susceptibility to chemotherapy. Significantly decreased glutathione content in tumor cells in the GLN + MTX group correlated with enhanced tumor volume loss (−0.8 ± 1.0 mL in GLN + MTX *vs.* +9.5 ± 2.0 mL in GLY + MTX, $p < 0.05$).

Conclusion

These data suggest that oral glutamine supplementation will enhance the selectivity of antitumor drugs by protecting normal tissues from and possibly sensitizing tumor cells to chemotherapy treatment-related injury.

Catabolic states such as major surgery, sepsis and cancer are characterized by alterations in the interorgan exchange of amino acids, net skeletal muscle breakdown, and negative nitrogen balance.[1-8] Toxicity to the tumor-bearing host is seen from the disease and from chemotherapy treatment-related injury. Dose intensification of chemotherapy is thought to increase survival.[9,10] However, the limitation of intensifying chemotherapeutic regimens has become the severity of toxicity to the normal host tissues.[11,12]

Glutamine (GLN) is a nonessential amino acid that serves as a primary respiratory fuel and also as a necessary substrate for nucleotide synthesis in most dividing cells.[13] Methotrexate-treated rats fed a glutamine-supplemented diet demonstrate improvements in gut toxicity, hematologic parameters, "sepsis," and survival.[14,15] Glutamine also is a principal fuel used by most rapidly proliferating tumors.[6] However, supplemental GLN supports host glutamine metabolism without stimulating tumor growth.[16] When given concomitantly with methotrexate (MTX), glutamine significantly enhances its tumoricidal effectiveness.[15] The mechanism of this differential toxicity is unknown, but it may be related to changes in glutathione (GSH) metabolism. Welbourne demonstrated that when the kidney is under oxidant stress, GLN is rate-limiting for GSH synthesis.[17] Glutathione is a potent ubiquitous antioxidant that also is an important factor in the metabolism of many drugs and endogenous substances.[18,19] An important function of GSH is protection of critical cellular molecules. Toxicity of target tissue is a result of depletion of tissue GSH concentration and protein alkylation.[19] We hypothesized that supplemental GLN may increase the therapeutic index of MTX by improving host tolerance through changes in GSH metabolism. This study examined the influence of supplemental oral GLN on tumor and host GSH metabolism and response to MTX in a rat sarcoma model.

MATERIALS AND METHODS

Animal Preparation and Diets

Male Fischer 344 rats (300 g) were obtained from SASCO Inc. (Omaha, NE). All studies were approved by the Animal Care and Use Committee at the John L. McClellan Veteran's Hospital. The rats were maintained in cages in the animal care facility. The rats were subjected to alternate 12-hour periods of dark/light cycle and given at least 1 week to acclimate to the animal care facilities. During that time, the rats were allowed *ad libitum* intake of standard rat chow and water. Animals were randomized during the study period to receive isonitrogenous isocaloric chow diets supplemented with 1 g/kg/day elemental GLN or glycine (GLY) by gavage.

Tumor Cell Implantation

After 1 week of acclimation to the animal care facility and on day 0 of the study, 36 rats were randomized to flank implantation of a $2 \times 2 \times 2$ mm^3 of viable methylcholanthrene-induced fibrosarcoma cells. This tumor model has been used previously by the author[7,15-16,20,21] to study tumor host metabolism interaction. This tumor-cell line is fast-growing and locally aggressive, metastasizes rarely, and never regresses spontaneously.

Study Procedure

On day 21 after tumor cell implantation, rats were randomized to receive pair-fed chow diets with supplemental GLN or GLY by gavage. On day 23, after 2 days of prefeeding, rats were randomized to one of the following four groups receiving an intraperitoneal injection of MTX (20 mg/kg) or saline (CON): GLN + MTX, GLY + MTX, GLN-CON, or GLY-CON. Each group contained nine rats. On day 24, all rats were weighed and anesthesia was obtained with ketamine (7.5 mg/100 g body weight) and acepromazine (0.1 mg/100 g body weight). Under sterile conditions, a mid-line incision was made, and the rat was heparinized. Arterial blood was withdrawn from the aorta using a 25-gauge needle attached to a 1-mL syringe. Blood was processed for arterial GLN content. The jejunum and kidney were removed and processed for glutaminase enzyme activity and GSH content. A portion of heart ventricle, gastrocnemius muscle, liver, and lung also were removed and processed for GSH content. The tumors were measured, weighed, and assayed for glutaminase activity and GSH content.

Processing of Samples

Aliquots of heparinized whole blood were mixed with equal volumes of cold 10% perchloric acid then vortexed and centrifuged at 5 C at $3000 \times G$ for 10 minutes. The supernatant was removed and neutralized with an equal amount of cold 0.48 M K_3PO_4. This was vortexed and centrifuged at 5 C at $3000 \times G$ for 10 minutes. The supernatant was removed and kept frozen at -20 C for later determination of GLN concentration by the microanalytical method described by Bergmeyer.[22]

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The tumor was removed from the flank. A 0.5-g portion of the tumor was homogenized immediately with 50 mmol sodium phosphate buffer with 300 mm sucrose for 1 minute on ice. An aliquot of this mixture then was removed for glutaminase activity and protein determination. Protein was determined by the Lowry method.[23] Phosphate-dependent glutaminase activity was determined using a microfluorometric assay similar to that described by Windmueller.[24] Another 0.5-g portion of tumor tissue was homogenized similarly in 5% 5-sulfosalicylic acid. The homogenate then was centrifuged for 5 minutes at 10,000 RPM, and the supernatant was used to determine total GSH content via a standard enzymatic recycling procedure, as described by Tietze and modified by Anderson.[25,26] For determination of GSH disulfide (GSSG), an aliquot of the previous supernatant was mixed with TRIS buffer and 2-vinyl pyridine (Aldrich Chemical Co., Milwaukee, WI) and assayed via the method of Griffith.[27]

Mucosa from a 5-cm section of proximal jejunum was scraped and homogenized in 50 mmol phosphate buffer containing 300 mmol sucrose, and phosphate-dependent glutaminase was determined immediately.[20] A second section was similarly scraped and homogenized in 5% 5-sulfosalicylic acid and assayed for GSH.[25] An aliquot of this was treated with 2-vinyl pyridine and TRIS buffer. These samples were stored at −80 C until assayed for total GSH and GSSG. Samples (0.5 g) of heart ventricle, liver, skeletal muscle, kidney, and lung were processed similarly and stored for total GSH and GSSG.

Calculations/Statistical Analysis

All data are expressed as mean ± standard error. Differences between means are considered significant at the $p < 0.05$ level, using analysis of variance. Statistical analyses were performed using a Macintosh IIci computer/StatView II (Apple Computers, Inc., Cupertino, CA/Abacus Concepts, Inc., Berkley, CA).

RESULTS

Body Weights and Food Intake

Chow intake was identical during the study period, as was the volume of gavage of GLN and GLY solution (10 mL/day). There were no significant weight differences between groups at initiation of the study or at death (319 ± 5 g in GLN + MTX *vs.* 327 ± 4 g in GLY + MTX *vs.* 326 ± 6 g in GLN-CON *vs.* 318 ± 11 g in GLY-CON, p = NS).

Arterial Glutamine Concentration

Control rats receiving supplemental GLN had elevated arterial GLN concentrations compared with GLY-CON (685 ± 10 μmol/L in GLN-CON *vs.* 501 ± 32 μmol/l in GLY-CON, $p < 0.01$). A similar difference was seen between groups receiving MTX (665 ± 19 μmol/L in GLN + MTX *vs.* 522 ± 27 μmol/L in GLY + MTX, $p < 0.01$). There was no difference in arterial GLN concentrations in similarly fed groups. Only the blood GLN in the glutamine-supplemented groups remains elevated above normal levels.

Table 1. EFFECTS OF A GLUTAMINE DIET ON TUMOR-GROWTH PARAMETERS

	Initial Tumor Volume (cc)	Tumor Volume Loss (cc)	Tumor Glutaminase Activity (μm/mg/min)
GLN + MTX	51.3 ± 6.3	−0.8 ± 1.0*	2.92 ± 0.19†
GLY + MTX	42.3 ± 6.3	+9.5 ± 2.0	5.43 ± 0.21
GLN-CON	51.9 ± 5.9	+5.7 ± 1.6	4.78 ± 0.13
GLY-CON	42.7 ± 5.8	+8.4 ± 1.8	4.48 ± 0.36

* $p < 0.05$, GLN + MTX *vs.* all others, analysis of variance. Amount of tumor loss from initial tumor volume 22 hours after methotrexate injection. Loss is represented by a negative number.
† $p < 0.05$ GLN + MTX and *vs.* all other groups.

Tumor Growth Parameters

Initial tumor volume was not significantly different among the four groups (Table 1). Tumor glutaminase activity was significantly decreased only in the GLN + MTX group (2.92 ± 0.19 μm/mg/min in GLN + MTX *vs.* 5.43 ± 0.21 μm/mg/min in GLY + MTX, $p < 0.01$). This correlated with significantly more tumor volume loss (−0.8 ± 1.0 cc in GLN + MTX *vs.* +9.5 ± 2.0 cc in GLY + MTX, $p < 0.05$).

Tissue Glutathione Levels

Provision of a GLN-enriched diet during MTX treatment decreased tumor GSH levels while increasing or maintaining host GSH stores (Table 2). In contrast, GLY-supplemented rats showed no change in GSH stores in tumor cells from control animals and remained significantly elevated above the GLN + MTX group. Significantly decreased GSH content in tumor cells in the GLN + MTX group correlated with enhanced tumor volume loss (Table 1). Host-tissue GSH levels in the GLY + MTX group were significantly lower than those seen in the GLN + MTX group. This correlated with higher tissue oxidant injury as measured by GSSG/GSH (Fig. 1). Oxidized GSH levels (GSSG) in the GLY + MTX group were significantly elevated above GLN +

Table 2. REDUCED TISSUE GSH CONTENT

	Tumor GSH +	Heart GSH +	Kidney GSH +	Gut GSH +	Lung GSH	Muscle	Liver GSH
GLN + MTX (n = 9)	2.38 ± 0.17*	1.83 ± 0.06†	2.21 ± 0.14†	2.60 ± 0.28†	2.06 ± 0.05	1.42 ± 0.12	6.16 ± 0.48
GLY + MTX (n = 9)	2.92 ± 0.20	1.69 ± 0.05	1.66 ± 0.05	1.93 ± 0.18	1.55 ± 0.05	0.86 ± 0.04	5.77 ± 0.26
GLN-CON (n = 9)	3.14 ± 0.13	1.86 ± 0.09	2.23 ± 0.14	3.37 ± 0.50	1.74 ± 0.05	1.33 ± 0.06	5.47 ± 0.28
GLY-CON (n = 9)	2.92 ± 0.20	1.99 ± 0.04	2.32 ± 0.18	2.32 ± 0.18	1.79 ± 0.07	0.813 ± 0.06	5.62 ± 0.46

* $p < 0.05$ *vs.* all other groups, analysis of variance, +GSH expressed as μm/g tissue.
† $p < 0.05$ *vs.* GLY + MTX, analysis of variance.

MTX in kidney, gut, liver, and muscle, although these did not differ from GLY-CON except in muscle and liver (Table 3). Total GSH in the GLN-MTX group was significantly elevated in all host tissues when compared with the GLY-MTX group (Fig. 2). This contrasted sharply with the significant decrease in total GSH content in the tumor cells of the GLN-MTX group when compared with all other groups (Fig. 2).

DISCUSSION

Previous work in our laboratory demonstrated a significantly enhanced tumoricidal effect of MTX in rats fed an elemental glutamine-supplemented diet. Improvements in gut toxicity, hematologic parameters, sepsis, and survival also were demonstrated.[15] One mechanism by which the provision of supplemental GLN to the sarcoma-bearing rat enhances the tumoricidal effect of MTX is by increasing the intracellular tumor concentration of MTX.[28] However, this is not the entire story. How does GLN protect the host against radiation therapy?[29–31] Why does GLN decrease MTX-related gut toxicity? Why does one patient tolerate chemotherapy or radiation therapy without complication and another suffer severe morbidity or even death?

We proposed that an alternative mechanism by which GLN can enhance chemotherapy toxicity is by alteration in GSH metabolism. Glutathione is a tripeptide that is ubiquitous and acts in a protective role against oxidant injury in normal tissue and as a resistance mechanism against radiation and chemotherapy-related injury in tumor tissue.[32] Welbourne demonstrated that when the kidney receives an oxidant stress, GLN becomes rate-limiting for GSH synthesis.[17] In addition, GSH plays a central role in calcium metabolism, leukotriene biosynthesis, thyroid metabolism, membrane and channel function, and nutrition.[19] Depletion of greater than 70% of GSH tissue is associated with irreversible cellular damage. The results of this study demonstrate that one mechanism by which supplemental GLN may increase

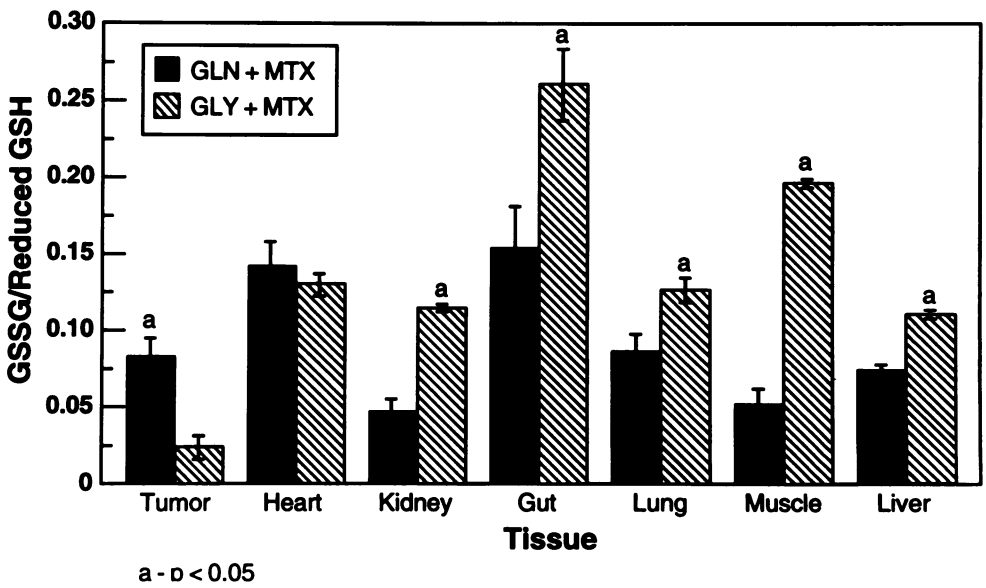


Figure 1. Oxidized:reduced glutathione ratio in rats supplemented with glutamine or glycine receiving MTX. This ratio is proportional to the amount of tissue oxident injury.

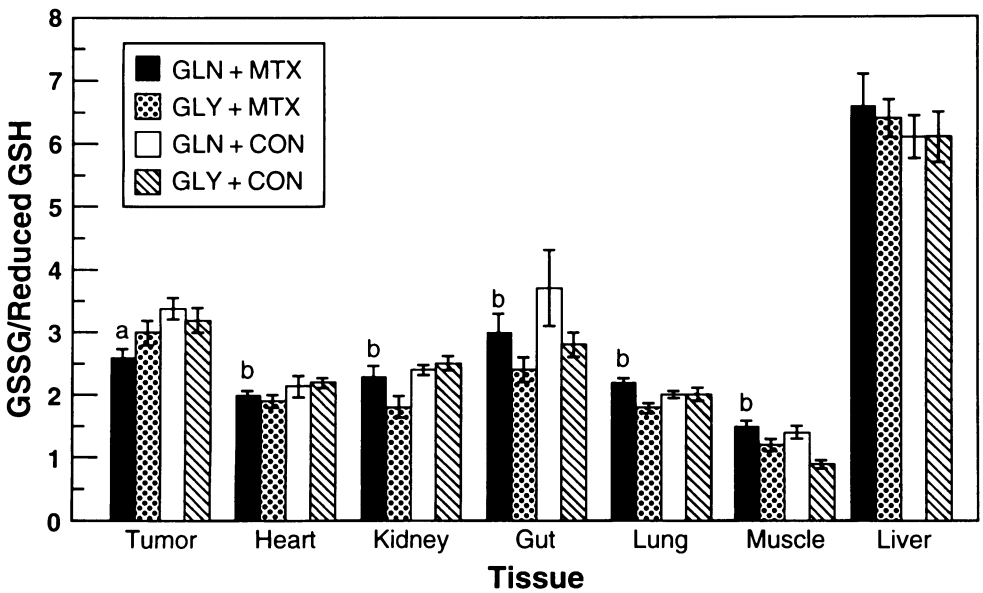
Table 3. OXIDIZED TISSUE GLUTATHIONE CONTENT

	Tumor GSSG+	Heart GSSG+	Kidney GSSG+	Gut GSSG+	Lung GSSG+	Muscle GSSG+	Liver GSSG+
GLN + MTX	0.19 ± 0.02*	0.27 ± 0.02	0.10 ± 0.02*	0.37 ± 0.04*	0.19 ± 0.01	0.07 ± 0.01*	0.46 ± 0.04*
GLY + MTX	0.08 ± 0.03	0.21 ± 0.02	0.19 ± 0.02	0.47 ± 0.04	0.20 ± 0.02	0.17 ± 0.02	0.61 ± 0.05
GLN-CON	0.20 ± 0.02	0.27 ± 0.01	0.11 ± 0.01	0.32 ± 0.03	0.23 ± 0.01	0.03 ± 0.01	0.48 ± 0.04
GLY-CON	0.12 ± 0.02	0.24 ± 0.08	0.19 ± 0.01	0.48 ± 0.03	0.18 ± 0.02	0.11 ± 0.03	0.41 ± 0.05

* $p < 0.05$ *vs.* GLY + MTX, analysis of variance, +GSSG expressed as μm/g tissue.

the therapeutic index of MTX and other therapies is by improving host tolerance through upregulation of host and downregulation of tumor GSH metabolism.[33]

Provision of supplemental oral GLN to the tumor-bearing host enhances the tumoricidal effect of MTX and is associated with a decrease in intracellular tumor GSH levels and decreased GLN metabolism. Reduced and total GSH content of the tumor was significantly decreased only in the GLN-MTX group, which correlated with significantly greater tumor loss and oxidant injury as measured by the ratio of GSSG to GSH (Fig. 1). Although MTX injection caused significant reduction in GSH content in most host tissues, GLN supplementation restored GSH levels to normal. Decreased host treatment related-injury was demonstrated by a reduced GSSG:GSH ratio. A possible explanation for this dichotomy in tumor and host tissues may be the ability of GLN to act as a γ-glutamyl acceptor (Fig. 3).[17] In doing so, GLN promotes the ability of γ-glutamyl transpeptidase to take up GSH molecules, which requires oxidation of an intracellular GSH molecule as well as the production of a γ-glutamyl-glutamine dipeptide.[34] This dipeptide may further promote the same reaction via recycling or be used by the conjugase enzyme to produce polyglutamated MTX. We propose that the pH-sensitive oxoprolinase enzyme is blocked in the tumor cell and cannot regenerate GSH, a process that also requires three adenosine triphosphate molecules.[35] The tumor cell is, by definition, a more acidotic environment than normal tissue, and this is exacerbated with injury. In normal cells, the presence of abundant quantities of GLN can bypass this enzyme block by upregulating the glutaminase or γ-glutamyl transferase enzyme.[17] Carbon for carbon, glutamine is just as efficient an energy source as glucose and also may supply adenosine triphosphate.[6,13] The tumor γ-glutamyl transferase cannot be upregulated by substrate, just as tumor glutaminase cannot. Upregulation of tumor enzyme activity occurs through gene amplifi-



a - $p < 0.05$ vs. all other groups, ANOVA, +GSH exppressed as um/g tissue
b - $p < 0.05$ vs. GLY + MTX, ANOVA

Figure 2. Total glutathione (oxidized plus reduced form) in rats supplemented with glutamine ± MTX. (A) $p < 0.05$ GLN + MTX *vs.* all other groups; (B) $p < 0.01$ GLN + MTX *vs.* GLY + MTX.

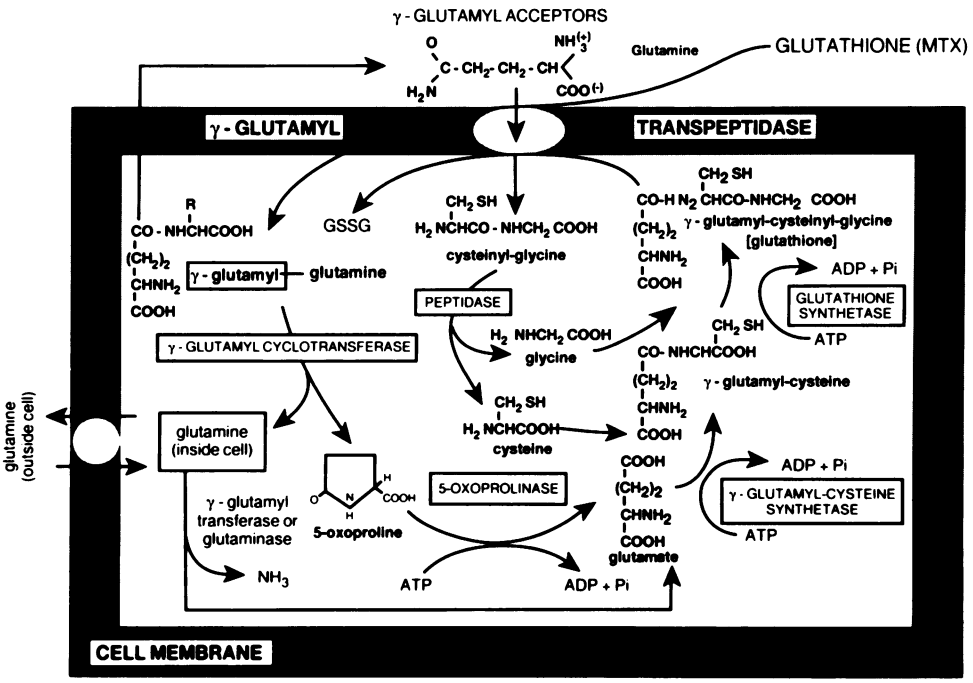


Figure 3. Proposed mechanism for increased host and decreased tumor intracellular glutathione levels in the presence of glutamine. In normal host tissues, GLN acts as a γ-glutamyl acceptor, promoting the uptake of host GSH and the breakdown of intracellular GSH. In host—but not tumor—tissue, GLN is able to bypass the bottleneck at the 5-oxoprolinase enzyme via γ-glutamyl transferase. GLN may also help supply energy for the obligatory adenosine triphosphate required for GSH synthesis.

cation. The fact that tumors containing high levels of γ-glutamyl transferase or glutaminase are more resistant to chemotherapeutic treatment further supports this hypothesis.[36] Significantly decreased glutaminase activity seen in the MTX + GLN group may decrease the supply of glutamate for GSH as well. The reason for the decrease in glutaminase enzyme activity is unclear because MTX is an antimetabolite and does not directly inhibit glutaminase enzyme activity. Decreases in GSH and glutaminase enzyme activity simply may reflect increased tumor-cell death.

Chemotherapeutic regimens are limited by the toxicity to host tissues. There is growing clinical evidence of the therapeutic value of high-dose chemotherapy regimens.[9,10] However, high-dose regimens have found limited use because of the severity of the associated toxicity.[12] Our studies suggest that GLN supplementation may not only increase MTX selectivity, but may be applicable to a wide variety of radiation and chemotherapies. Understanding the mechanisms that control the toxic dose thresholds for different therapies is an extremely important objective of research. The fundamental principles in host and tumor GLN and GSH metabolism may guide the attempt to safely use therapies already available and the development of new therapeutic regimens.

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